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Effect of hyperbarically oxygenated-perfluorochemical with UW solution on preservation of rat small intestine using an original pressure-resistant portable apparatus

Kazunori Inuzuka, MD, Naoki Unno, MD,FACS, Naoto Yamamoto, MD, Daisuke Sagara, MD, Minoru Suzuki, MD, Motohiro Nishiyama, MD, and Hiroyuki Konno, MD

Second Department of Surgery, Hamamatsu University School of Medicine

1-20-1 Handayama, Hamamatsu, Shizuoka, Japan 431-3192

Address for correspondence:

Naoki Unno, M.D.

Second Department of Surgery,

Hamamatsu University School of Medicine, 1-20-1 Handayama,

Hamamatsu, Shizuoka, Japan 431-3192

Telephone number: 81-(0)53-435-2279 Fax number: 81-(0)53-435-2273

E-mail address: unno@hama-med.ac.jp

Abstract

Background. Perfluorochemicals (PFC) are chemical substances that have a higher oxygen solubility under hyperbaric oxygen (HBO) pressure. This study investigated the effect of cold HBO-PFC/UW solution on preservation of rat small intestinal graft.

Methods. We manufactured an air-tight, pressure resistant tank which was made of stainless steel with high thermal conductivity. Rat ileal grafts were placed in a custom-made silicon-gum bag with UW solution, which was immersed in 5 atm HBO-PFC solution in the tank (Group P-5). The tank was placed at 4 ºC. We compared the ATP concentration and mucosal permeability in Group P-5 with grafts preserved in 1 atm oxygenated-PFC/UW solution (Group P-1) and simple cold storage in UW solution (Group C). Histological study was also performed.

Results. PO_2 in UW solution after 48 hours preservation were 1851.6 ± 36.9, 498.5 ± 12.5, and 173.3 ± 3.4 mmHg (Group P-5, P-1 and C, respectively, mean ± SD). At that time, graft ATP concentration was significantly higher in Group P-5 compare to that in Group P-1 and Group C. Mucosal hyperpermeability as well as mucosal morphological changes were also ameliorated in the Group P-5.

Conclusion. HBO-PFC could supply a larger amount of oxygen to UW solution. Indirect measures of oxygen metabolism such as ATP content and lactate production

suggested improvement in maintaining graft oxygen metabolism.

Key words: small intestine, perfluorochemical, hyperbaric oxygen, transplantation, UW solution

Abbreviations:

ATP: adenosine 5'-triphosphate

KHBB: Krebs-Henseleit bicarbonate buffer

HBO: hyperbaric oxygen

PFC: perfluorochemical

PO_2: partial oxygen pressure

UW solution: University of Wisconsin solution

Introduction

Recently, intestinal transplantation has become clinically feasible. However, the outcome is not sufficiently satisfactory compared to that after transplantation of other organs such as the kidney and liver. The small intestine induces not only strong immunoreaction after transplantation to another individual (*1*), but also mucosal hyperpermeability due to ischemia-reperfusion injury (*2*). Especially, derangement of the mucosal barrier function is reported to be inversely related to mucosal tissue ATP concentration (*2*). Therefore, maintenance of tissue oxygen metabolism is important to gut mucosal integrity. It is likely that an extension of preservation period will enable longer distance transportation of grafts, better selection of recipients and allow a longer period to prepare for transplantation surgery.

A simple cold storage method with University of Wisconsin (UW) solution has clinically been applied as a standard intestinal preservation (*3*). However, several experiments reported that the method was effective only for 24 hours preservation of the small intestine (*4*)(5). Recently, the cavity two-layer method using UW and Perfluorochemical (PFC) with continuous oxygen bubbling succeeded in preserving rat small intestine for 48 hours (*5*). In that method, PFC was used as an oxygen source to oxygenize UW solution with simple diffusion. However, this method requires

continuous oxygen bubbling in PFC solution to maintain the initial oxygen pressure, which is not considered practical for clinical use (6). Moreover, simple oxygen bubbling (i.e. under 1 atm) limits the oxygenation to no more than 600 - 700 mmHg, and 300 - 400 mmHg in PFC and UW solution, respectively (*7*). However, PFC can dissolve more oxygen under higher oxygen atmosphere. To utilize hyperbaric oxygen for oxygenizing PFC, we manufactured an original pressure-resistant portable apparatus to prepare hyperbarically-oxygenated PFC (HBO-PFC) solution, which can supply oxygen to UW solution constantly and thus provide the organ/tissue with sufficient oxygen. In this study, we introduced an original preservation method with cold HBO-PFC/UW solution for rat small intestine and assessed its protective effect.

MATERIALS AND METHODS

Original apparatus and storage method

We manufactured a pressure-resistant portable apparatus (cylindrical shape with a diameter of 20 cm, height of 20 cm and capacity of 6.3L, 3.5 kg) for preservation of organ grafts (Fig.1). This apparatus is made of stainless steel, air-tight, and can be sustain pressure up to 7 atm. Air can be deflated from the upper air cock. Before graft storage, 4L of PFC (Perfluorodecaline; F2 Chemicals Ltd., Lancashire, UK) was hyperbarically oxygenated at the desired pressure atmosphere inside the tank, while monitoring with a pressure gauge. Then the tank was placed in a conventional refrigerator to keep the temperature inside the tank at 4 ºC, which was monitored with a thermometer for sixty minutes. A custom-made silicon-gum bag (0.1 mm thick) with 100 ml UW solution (ViaSpan; Bistrol-Myers Squibb Co., USA) was immersed into the PFC solution in preparation for graft harvesting. Then, the pressure-resistant tank was deflated and was opened. Immediately after graft harvesting, the graft was packed in the silicon-gum bag and the bag was immersed in the PFC solution in a small inner lid. Thereafter, the tank was closed and re-oxygenized at the desired atmosphere. The tank was placed in the refrigerator at 4 ºC.

Determination of oxygen-permeable bag for graft storage

Before the graft storage experiments, we tested various types of bags to determine the oxygen permeability. We tested silicon-gum bags those were 0.1 mm thick, 0.2 mm thick (Keiju; Mitsubishi Jushi Co., Japan) and a polyethylene bag 0.07 mm thick (Ziploc; Asahi Kasei Life & Living Corp., Japan) by measuring the partial oxygen pressures (PO_2) in UW solution after immersing for 60 minutes in 5 atm HBO-PFC solution (Fig.2A). When the tank was opened, the PO_2 in the UW solution and HBO-PFC solution in the tank were measured (n = 5 / group).

Experimental animals and protocol

This study was approved by the Institutional Animal Care and Use Committee of the Hamamatsu University School of Medicine and followed the National Institutes of Health guidelines for treatment of laboratory animals. Male Sprague-Dawley rats weighing 250 to 350 g (SLC, Hamamatsu, Japan) had free access to food and water before the experiment. They were anesthetized by intramuscular injection of sodium pentobarbital (50 mg/kg). After a midline laparotomy, a 5-cm or 10-cm segment of the distal ileum (5 cm distant from the ileocecal valve) was isolated without mesentery, and

the intestinal lumen was cleaned with saline repeatedly. The grafts were randomly assigned to the following three groups. Group P-5: The silicon bag including intestinal grafts in 100 mL UW solution was immersed in 5 atm HBO-PFC. Group P-1: The silicon bag including intestinal grafts in 100 mL UW solution was immersed in 1 atm oxygenated-PFC. Group C : As a control, intestinal grafts were immersed in 100 mL UW solution in the silicon bag, which was stored inside the tank without oxygenation. In all of the groups, the tank was placed at 4 ºC. During preservation, the temperature inside the tank was monitored by a thermometer. Grafts were evaluated at the following time-points. 0 hour: Immediately after harvesting without storage, 12 hours: half a day (12 hours) preservation, 24 hours: 1 day (24 hours) preservation, 48 hours: 2 days (48 hours) preservation, 72 hours: 3 days (72 hours) preservation.

Measurement of tissue ATP content

Immediately after harvest, ileal grafts measuring 5 cm in length were frozen in liquid nitrogen and lyophilized overnight (n = 20 / group). Intestinal ATP levels were determined using a modification of the luciferin / luciferase method (CytoPro HTS Kit, Thermo Labsystems, Finland) (*2*). Bioluminescence measurements were subsequently performed using a luminometer (LUMI-COUNTER 700; MICROTECH Co.,LTD.,

Japan). The protein concentration was determined using the BCA (bicinchoninic acid) method with UV-VIS spectrophotometer (UV 2450; Shimadzu Co., Japan) according to the manufacturer's instructions. ATP data were calculated and expressed as nanomoles of ATP per mg protein.

Measurement of intestinal mucosal permeability

After the graft (10 cm length) harvest in different set of animals, mucosal-to-serosal permeability was measured in grafts of each group. Permeability of the fluorescent isothiocyanate dextran with a molecular weight of 4000 Da (FD4) was determined using an everted gut sac method (n = 10 / group) (2)(8-9). Everted gut sacs were prepared in ice-cold modified Krebs-Henseleit bicarbonate buffer (KHBB, pH 7.4). One end of the gut segment was ligated with a 4.0 silk. The segment was then everted onto a thin plastic rod, and the resulting everted gut sac was secured with a 4.0 silk suture to the grooved tip of a 2.5 mL plastic syringe containing KHBB. The everted gut sac was gently distended by injecting 1.5 mL of KHBB. The everted gut sac was then suspended in a 50-mL beaker containing 40 mL of KHBB with FD4 (20 μg/mL). The solution in the beaker was temperature jacketed at 37 ºC and was continuously bubbled with a gas containing 99.9% O_2. A 1.0-mL sample was taken from the beaker prior to

adding the everted gut sac in order to determine the initial external (i.e., mucosal surface) FD4 concentration. The everted gut sac was incubated for 30 min in KHBB solution containing FD4. The length of the gut sac was then measured. Fluid was aspirated from the serosal side to determine the FD4 concentration. The serosal and mucosal samples were centrifuged for 10 min at 1000*g*. Three hundred microliters of the supernatant was diluted with phosphate-buffered saline (PBS, 2.7 mL). Fluorescence was measured using a fluorescence spectrophotometer (FP 777; Japan Apectroscopic Co., LTD., Japan) at an excitation wavelength of 492 nm (slit width = 2.5 nm) and an emission wavelength of 515 nm (slit width = 10 nm). Permeability was expressed as the mucosal-to-serosal clearance of FD4 calculated using the following equations:

$$M = ([FD4]_{ser}) \times 1.5$$

$$F = M/30\ min$$

$$C = (F/[FD4]_{muc})/L$$

where M is the mass (in nanograms) of FD4 in the gut sac at the end of the 30 min incubation period, $[FD4]_{ser}$ is the FD4 concentration in the serosal fluid aspirated from the sac at the end of the 30 min incubation period, F is the flux of FD4 (in nanograms per minute) across the mucosa, $[FD4]_{muc}$ is the FD4 concentration measured in the beaker at the beginning of the 30 min incubation period, L is the length (in centimeters)

of the gut sac, and *C* is the clearance of FD4 (in nanoliters per minute per centimeter) across the mucosa.

Histological analysis

At time-points 12 hour, 24 hours, 48 hours and 72 hours, morphological changes after ischemic storage in Group P-5, Group P-1 and Group C were assessed. The grafts were fixed in 10% buffered formalin (pH 7.0) until processing for histologic sections (n = 5 / group). The sections were stained with hematoxylin-eosin stain. A blind histological evaluation was performed on sample slides without group numbers. Intestinal mucosal damage was graded on a six-tiered scale as defined by Chiu et al. in a minimum of 20 separate locations on each section (*10*). The grades are as follows: grade 0, normal mucosal villi; grade 1, development of subepithelial space; grade 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; grade 3, massive epithelial lifting down the side of the villi; grade 4, denuded villi with lamina propria and dilated capillaries exposed; and grade 5, digestion and disintegration of the lamina propria, hemorrhage, and ulceration.

Measurement of PO_2, PCO_2, pH and Lactic acid in UW solution

To evaluate changes in PO_2, PCO_2, pH and Lactic acid in UW solution, samples were analyzed at each time-point using a commercial blood gas analyzer (n = 5 / group) (Chiron 860; Chiron, Japan).

The effect of exchange of UW solution every 24 hours on graft tissue ATP concentration and mucosal permeability

To assess the influence of changes in UW solution during prolonged storage (such as accumulation of lactate), we compared graft tissue ATP concentration and mucosal permeability between grafts with UW solution that was not exchanged for 72 hours and grafts with freshly-prepared UW solution every 24 hours using 5 atm HBO-PFC. After 72 hours preservation, tissue ATP concentration (n = 20 in each) and intestinal mucosal permeability (n = 10 in each) were measured.

Data analysis

All data are expressed as the mean ± SD. One-way or two-way analysis of variance (ANOVA) followed by Fisher's PLSD test was performed to test differences in the mean values of tissue ATP concentration, intestinal mucosal permeability, histopathological findings, and parameters in UW solution between groups. Analysis of

the effect by exchanging UW solution was performed using Mann-Whitney's U test. $P < 0.05$ was considered significant.

Results

Measurement of PO_2 in UW solution in various types of bag

After immersing for 60 minutes in 5 atm HBO-PFC solution (PO_2: 3852.9 ± 50.8 mmHg, n = 5), PO_2 in the UW solution using silicon-gum bags 0.1 mm thick was 2019.5 ± 65.1 mmHg, which was significantly higher than those in UW solution using silicon-gum bags 0.2 mm thick (1671.5 ± 59.9 mmHg, n = 5, $P < 0.001$) or using polyethylene bags (1607.1 ± 52.3 mmHg, n = 5, $P < 0.001$) (Fig.2B). Therefore, we used the silicon-gum bags 0.1 mm thick.

Changes in graft tissue ATP concentration in 5 atm HBO-PFC group (Group P-5) until 72 hours

To demonstrate the superiority of HBO-PFC, we measured the changes in tissue ATP concentration in Group P-5 at 12 hours, 24 hours, 48 hours and 72 hours and compared the findings with those in Group P-1 and Group C at the same time-points (n = 20 in each group and time-point). At 12 hours, the ATP concentration in Group P-5 was 71.7 ± 35.4 nmol/mg protein, which was significantly higher than that in either Group P-1 (58.3 ± 28.6 nmol/mg protein, P = 0.038) or Group C (31.8 ± 18.4 nmol/mg

protein, $P < 0.001$) (Fig. 3). At 24 hours, the ATP concentration in Group P-5 was 57.3 ± 36.3 nmol/mg protein, which was significantly higher than that in either Group P-1 (26.6 ± 8.3 nmol/mg protein, $P < 0.001$) or Group C (13.0 ± 11.0 nmol/mg protein, $P < 0.001$). Also, at 48 hours, the ATP concentration in Group P-5 was 34.9 ± 33.8 nmol/mg protein, which was significantly higher than that in either Group P-1 (21.9 ± 8.1 nmol/mg protein, $P = 0.044$) or Group C (3.8 ± 4.6 nmol/mg protein, $P < 0.001$). However, after 72 hours preservation, the ATP concentration in Group P-5 was decreased to 7.1 ± 2.8 nmol/mg protein, which was not significantly higher than that in either Group P-1 (8.6 ± 3.4 nmol/mg protein, $P = 0.82$) or Group C (2.5 ± 0.7 nmol/mg protein, $P = 0.48$).

Changes in graft mucosal permeability in 5 atm HBO-PFC group (Group P-5) until 72 hours

We measured the changes in mucosal permeability in Group P-5 at 12 hour, 24 hours, 48 hours and 72 hours and compared the findings with those in Group P-1 and Group C at the same time-points (n = 10 in each group and time-point). After 12 hours preservation, the permeability in Groups P-5, P-1 and C was 11.7 ± 4.7 nL/min/cm, 21.1 ± 3.2 nL/min/cm and 32.6 ± 11.3 nL/min/cm, respectively (Fig. 4). Mucosal

hyperpermeability in Group P-5 was significantly inhibited compared to those in Group P-1 ($P = 0.041$) and Group C ($P < 0.001$). After 24 hours and 48 hours preservation, the mucosal hyperpermeability was significantly inhibited in Group P-5 (19.0 ± 7.0 nL/min/cm and 29.6 ± 4.8 nL/min/cm, respectively) compared to Group C (34.8 ± 13.8 nL/min/cm and 48.6 ± 11.2 nL/min/cm, respectively, $P < 0.001$). On the other hand, although the mucosal permeability at 24 hours in Group P-1 (23.6 ± 7.6 nL/min/cm) was higher than that of Group P-5, there was no significant difference between the two groups ($P = 0.30$). However, at 48 hours, the mucosal hyperpermeability was significantly inhibited in Group P-5 compared to Group P-1 (38.9 ± 6.6 nL/min/cm, $P = 0.041$). After 72 hours preservation, the permeability in Groups P-5, P-1 and C was 44.0 ± 14.5 nL/min/cm, 42.9 ± 5.6 nL/min/cm and 50.9 ± 9.0 nL/min/cm, respectively. Mucosal hyperpermeability in Group P-5 was not significantly inhibited compared to those in Group P-1 ($P = 0.81$) and Group C ($P = 0.13$).

Histological analysis

At time-points 12 hour, 24 hours, 48 hours and 72 hours, morphological changes after ischemic storage in Group P-5, Group P-1 and Group C were assessed ($n = 5$ in each group and time-point). In Group C, subepithelial space developed and extended

with moderate lifting of the epithelial layer from the lamina propria at 12 hours. At 72 hours, denuded villi with lamina propria or disintegration of the lamina propria was observed. However in Group P-5, the graft did not show an extension of the subepithelial space and well preserved villi structures even at 72 hours. Histological evaluation by Chiu's score demonstrated that histological damage to the graft in Group P-5 was significantly less than that in Group C or Group P-1 at each stage (Fig. 5). Chiu's scores in Group P5 were 0.4 ± 0.5, 0.8 ± 0.4, 1.6 ± 0.5 and 2.0 ± 0.7 at 12 hours, 24 hours, 48 hours and 72 hours, respectively, while those in Group P-1 were 1.2 ± 0.4 ($P = 0.020$), 1.6 ± 0.5 ($P = 0.020$), 2.4 ± 0.5 ($P = 0.020$) and 3.6 ± 0.5 ($P < 0.001$), and those in Group C were 1.8 ± 0.4 ($P < 0.001$), 2.4 ± 0.5 ($P < 0.001$), 3.2 ± 0.4 ($P < 0.001$) and 4.8 ± 0.4 ($P < 0.001$).

Measurement of PO_2, PCO_2, pH and Lactic acid in UW solution

Figure 6 and Figure 7 show the changes in PO_2, PCO_2, pH and lactate in UW solution in silicon bag at 12 hours, 24 hours, 48 hours and 72 hours in Group P-5, Group P-1 and Group C (n = 5 in each group and time-point). After 24 hours preservation, PO_2 in UW solution in Group P-5 was slightly decreased compared to the initial level (0 hour), but remained to be around 2000 mmHg (1942.0 ± 57.2 mmHg and

2019.5 ± 65.1 mmHg, 24 hours and 0 hours, respectively, P = 0.06). In Group P-5, PO_2 in UW solution at 72 hours was 1814.0 ± 84.7 (P < 0.001) mmHg, which showed that the decrease in PO_2 over 72 hours was very subtle. On the other hand, at 0 hour, PO_2 in UW solution in Group P-1 and Group C was 632.6 ± 20.5 mmHg and 202.2 ± 9.5 mmHg, respectively. At 12 hours, PO_2 in UW solution in both groups was significantly decreased compared to the initial level (577.5 ± 17.4 mmHg and 188.2 ± 10.7 mmHg, P < 0.001 and P = 0.011, respectively). PO_2 in UW solution in both groups was gradually decreased during 72 hours preservation (473.8 ± 18.0 mmHg and 166.9 ± 5.8 mmHg, P < 0.001 and P < 0.001, respectively). With regard to PCO_2, at 0 hour, there was no significant difference among Groups P-5, P-1 and C (1.8 ± 0.5 mmHg, 1.8 ± 0.3 mmHg and 2.0 ± 0.3 mmHg, respectively). However, as preservation was prolonged, PCO_2 in UW solution in Group P-5 increased to 4.2 ± 0.7 mmHg, 4.8 ± 0.4 mmHg, 5.2 ± 0.6 mmHg (24 hours, 48 hours, and 72 hours, respectively), which was significantly higher than that in either Group P-1 (3.4 ± 0.5 mmHg, P = 0.007, 3.8 ± 0.3 mmHg, P < 0.001, 3.9 ± 0.3 mmHg, P < 0.001) (24 hours, 48 hours, and 72 hours, respectively) or Group C (2.6 ± 0.3 mmHg, P < 0.001, 2.7 ± 0.2 mmHg, P < 0.001, 2.8 ± 0.3 mmHg, P < 0.001) (24 hours, 48 hours, and 72 hours, respectively).

With regard to pH of the UW solution, all groups showed stable pH throughout the

preservation periods. On the other hand, the level of Lactate in UW solution steadily increased at 48 hours and 72 hours in each group. However, the elevation was significantly larger in Group C than that in Group P-5 at 48 hours and 72 hours (D2: 0.9 ± 0.2 mmol/L and 1.6 ± 0.3 mmol/L $P < 0.001$, D3: 1.5 ± 0.2 mmol/L and 2.1 ± 0.2 mmol/L $P < 0.001$, Group P-5 and Group C, respectively). Also, the elevation was significantly larger in Group P-1 than that in Group P-5 at 48 hours and 72 hours (D2: 1.5 ± 0.1 mmol/L $P < 0.001$, D3: 1.9 ± 0.2 mmol/L $P = 0.030$, Group P-5 and Group C, respectively).

The effect of exchanging the UW solution every 24 hours on graft permeability and ATP concentration

With 5 atm HBO-PFC, ATP concentration (n = 20 / in each group and time-point) and mucosal permeability (n = 10 / in each group and time-point) were compared at 72 hours between the grafts with unexchanged UW solution and those with exchanged solution. There were no significant differences in either parameter between the groups. (ATP: 8.1 ± 3.5 nmol/mg protein and 7.1 ± 2.8 nmol/mg protein, UW unexchanged and UW exchanged groups, respectively, $P = 0.32$, mucosal permeability: 40.8 ± 14.5 nL/min/cm and 44.0 ± 14.5 nL/min/cm, UW unexchanged and UW exchanged groups,

respectively, P = 0.63).

Discussion

Simple storage of grafts in cold UW solution is currently one of the standard preservation methods for small bowel transplantation. To improve the oxygen availability, the cavity two-layer (UW/PFC) cold storage method was introduced, in which UW solution was used to provide the energy ingredient for grafts and PFC was the oxygen provider separated from the upper layer of the UW solution because it is hydrophobic and has a higher specific gravity (*11*). PFC is an inert and excellent oxygen carrier and possesses an anti-oxidant effect (*12, 13*). It has been clinically applied as an artificial blood or for liquid ventilation (*14, 15*). Experimental studies demonstrated that intraluminal administration or peritoneal lavage with oxygenated PFC (oxy-PFC) showed excellent intestinal mucosal preservation in intestinal ischemia-reperfusion models (*16-18*). We recently demonstrated that peritoneal lavage with oxy-PFC protects the intestinal mucosa and increases survival in a hemorrhagic shock-resuscitation model in rats (*19*). In these experiments, oxygen derived from oxy-PFC diffuses to the intestinal mucosa via the serosa and protects ischemic tissue by maintaining oxygen metabolism. However, without continuous oxygen bubbling, PFC steeply loses its dissolved oxygen within a few hours in accordance with the pressure gradient (*20*). Practically, equipment with an oxygen cylinder for continuous oxygen bubbling makes

the storage apparatus more complicated and less portable. However, PFC can dissolve more oxygen under higher oxygen pressure in a linear fashion, i.e. HBO-PFC can carry more oxygen. With an original, air-tight, and pressure-resistant tank in combination with an oxygen permeable silicon-gum bag, our HBO-PFC/UW method facilitates storage with both high oxygen availability and excellent portability. Because the tank is made of stainless steel with high thermal conductivity, hypothermic graft preservation can easily be achieved by placing the apparatus in a conventional refrigerator or ice box. Although we manufactured the apparatus to preserve a rat small intestine, it may be applicable to other types of organs and tissue, such as pancreas (islet) and kidney. To apply this method to human bowel transplantation, a larger tank that is approximately three times the size of this apparatus may be need. We think that it would be technically feasible to fabricate such a larger tank.

In this study, silicon-gum bag 0.1mm thick showed excellent oxygen permeability to maintain PO_2 in UW solution around 2000 mmHg after being immersed in 5 atm HBO-PFC. Moreover, our air-tight, pressure-resistant tank maintained high PO_2 in both PFC and UW solution for 3 days. Using 5 atm HBO-PFC/UW solution (Group P-5), rat ileal grafts showed higher ATP concentrations and amelioration of mucosal hyperpermeability as well as better histological grading than grafts stored using the 1

atm oxy-PFC/UW method (Group P-1) for 48 hours. It is likely that the larger amount of oxygen delivered from HBO-PFC could enhance tissue preservation. To determine whether there is an incremental benefit of 2 atm, 3 atm, 4 atm and 5 atm oxygenation, further study is needed.

The preservation of tissue ATP is considered a reflection of graft viability and maintenance of tissue oxygen metabolism. The decreased level of ATP during ischemia is proportional to the degree of tissue injury by the oxygen-free radicals after reperfusion (*21, 22*). Moreover, the relationship between tissue ATP and mucosal permeability has been studied. Using intestinal epithelial cell monolayers, paracellular permeability via tight junction was demonstrated to increase in correlation with the degree of ATP reduction during prolonged hypoxia, metabolic inhibition and acidosis (*23, 24*). Intestinal mucosal hyperpermeability after ischemia-reperfusion is inversely related with mucosal tissue ATP concentration in a rat model (*6*). Therefore, ATP concentration is thought to be a determinant of intestinal barrier function. Indeed, reduction of ATP concentration in graft tissue was paralleled with mucosal hyperpermeability in this study. Derangement of mucosal barrier function is associated with increase in transmucosal movement of bacteria, lipopolysacharide, and various hydrophilic solutes (*25, 26*) and being considered to be the pathogenesis of hemorrhagic

shock and multiple organ dysfunction syndrome (MODS) (*26, 27*). Therefore, it seems very important to preserve gut mucosa and keep the barrier function in small bowel transplantation (*28, 29*).

In this study, we prolonged the preservation period up to 72 hours using 5 atm HBO-PFC/UW solution (Group P-5) and tested graft viability by assessing the ATP concentration, mucosal permeability and histological findings. At 12 hours, that is currently considered as a limit in clinical practice, the cold HBO-PFC/UW method succeeded in preserving the graft superiorly according to the changes in the parameters obtained in comparison with 1 atm oxy-PFC/UW method (Group P-1) or simple cold UW storage (Group C). However, the cold HBO-PFC/UW method showed better preservation effects until 48 hours, but failed for 72 hours compared to the other groups. PCO_2 in UW solution gradually increased after preserving grafts. Elevations in Group P-5 were significantly higher than those in Group P-1 or Group C at 24, 48, and 72 hours. Similarly, elevations in Group P-1 were significantly higher than those in Group C at the same time points. However, elevations of lactate concentration in the UW solution in Group P-5 was significantly inhibited compare to those in Group P-1 or Group C at 48 and 72 hours. Taken together, grafts in Group P-5 might maintain aerobic respiration better than those in Group P-1 or Group C.

We suspected that lactate or unknown substances that accumulated in the UW solution may have perturbed the oxygen metabolism. Therefore, we performed the additional experiments to compare the graft viability between grafts with unexchanged UW solution and those with exchanged UW solution every 24 hours. As was mentioned in results section, there was no beneficial effect of exchanging UW solution. This suggested that accumulated substances in the UW solution during the prolonged preservation did not account for the loss of tissue ATP content and mucosal hyperpermeability. Therefore, we considered that the graft developed a failure of oxygen metabolism despite the ample availability of both oxygen and energy source for unknown reasons. The pathogenesis may involve the occurrence of so-called cytopathic hypoxia. Cytopathic hypoxia is an acquired intrinsic defect in cellular respiration considered associated with MODS, in which anaerobic metabolism is accelerated despite increased organ perfusion or supranormal oxygen delivery (*30*). The proposed substances that damage cellular mitochondrial respiratory function are various cytokines, nitric oxide, poly-(ADP-ribosyl)-polymerase (PARP), and reactive oxygen species (*31-33*). However, previous studies demonstrated that HBO increases reactive oxygen species formation and causes cellular (organ) damage (*34-36*). The intestine is known to be vulnerable to oxidant stress *(37)*. Taken together, the prolongation of storage in

HBO-PFC/UW solution might cause oxidative damage to the graft mitochondria, which might result in cytopathic hypoxia. Further study is needed to investigate this speculation. Assessment of tissue lipid peroxidation with/without free radical scavengers may clarify the mechanism. Identification of the most effective HBO pressure to maintain a balance between oxygen availability and oxidative stress remains to be elucidated.

In conclusion, we manufactured an air-tight, pressure resistant tank made of stainless steel with high thermal conductivity, and assessed the preservation effect of cold HBO-PFC/UW solution using an oxygen-permeable silicon-bag. This original method enhanced the preservation of rat small intestine in terms of tissue oxygen metabolism and mucosal permeability as well as histological findings. Moreover, this method allowed an excellent portability of the preserved graft. However, a limitation of this study is that it remains to be determined whether the improvement of the parameters above is associated with a better function in transplanted graft. Therefore, we are planning to extend this study to assess graft function using a transplantation model in the future. Similarly, further study is needed to determine the optical HBO pressure to extend the preservation period. Then, this method may be applicable to small bowel transplantation in clinical practice.

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LEGENDS

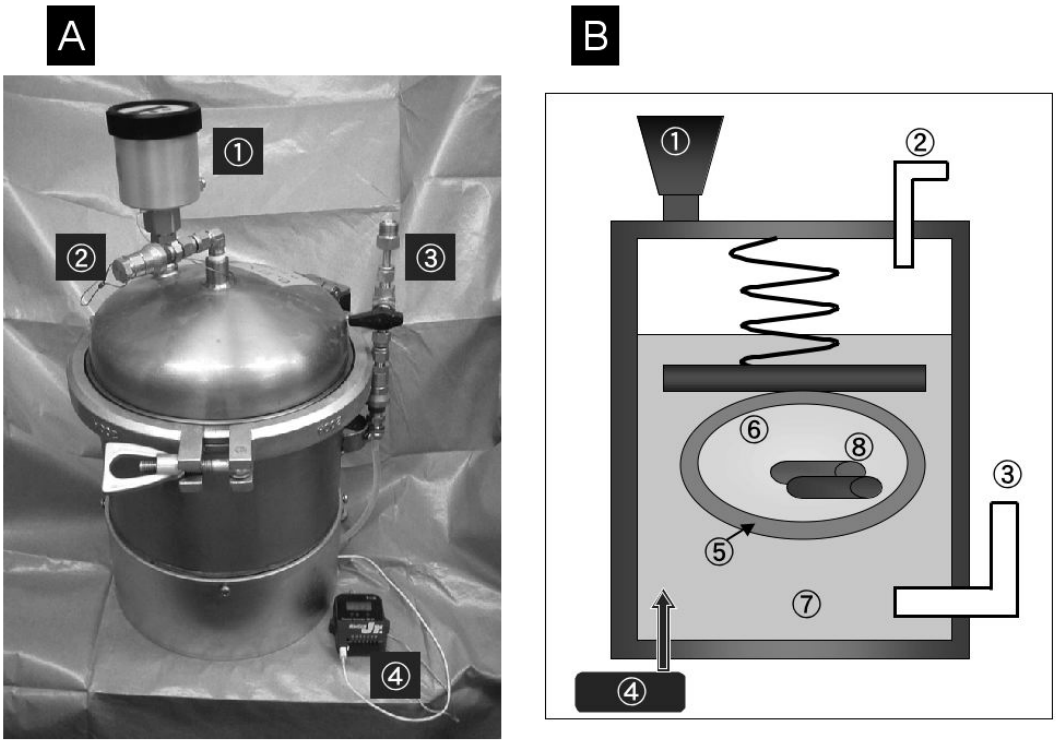


Figure 1.

The original air-tight, pressure-resistant portable apparatus for preservation of the organ. (1) pressure gauge; (2) air cock for deflation; (3) air cock for inflation; (4) thermometer; (5) silicon-gum film bag; (6) University of Wisconsin solution; (7) perfluorochemical solution ; (8) small intestine grafts

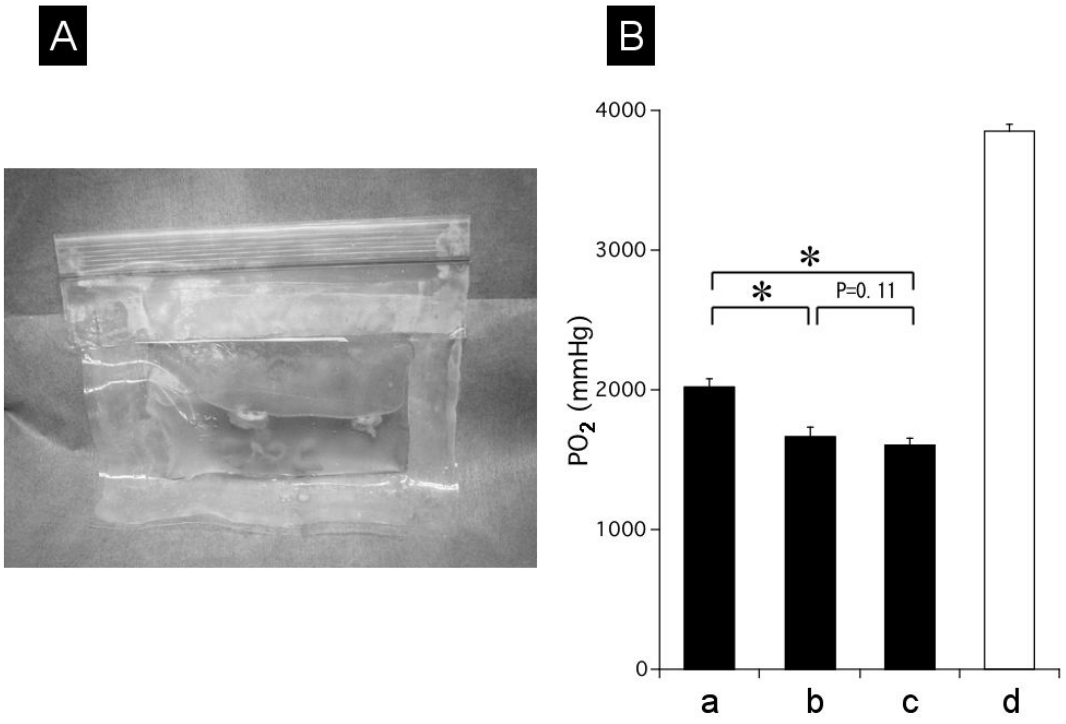


Figure 2.

(A) The grafts in UW solution packed in custom-made silicon-gum bag.

(B) PO_2 in UW solution packed with bags made of (a) silicon-gum film 0.1 mm thick, 0.2 mm thick (b), or 0.07 mm polyethylene film (c) in 5 atm HBO-PFC for 60 minutes. The PO_2 level in 5 atm HBO-PFC (d) was also measured (n = 5 / group).

* $P < 0.05$

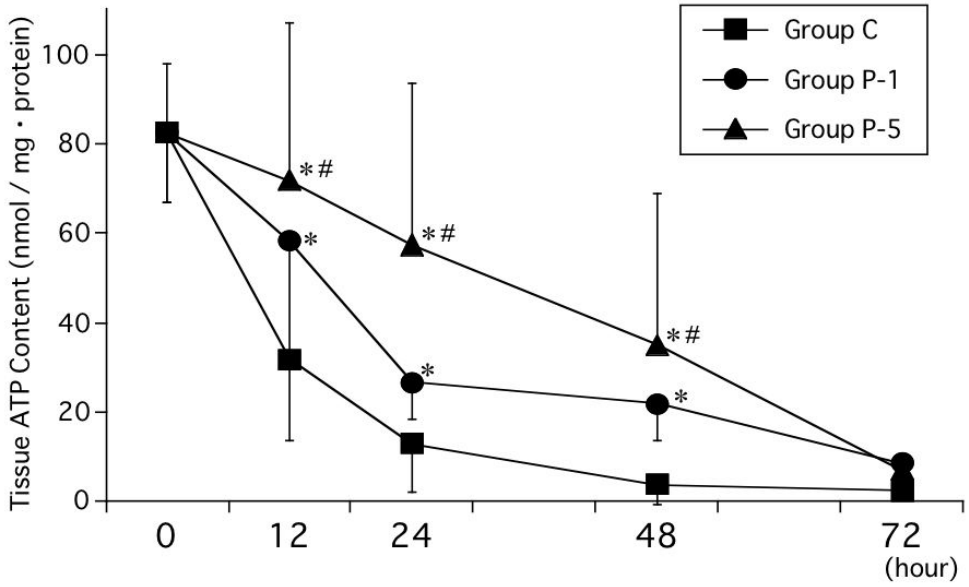


Figure 3.

Changes in tissue ATP concentration in 5 atm HBO-PFC/UW (Group P-5), 1 atm O2-PFC/UW (Group P-1) and simple cold UW storage (Group C) until 72 hours (n = 20 in each group and time-point). The ATP concentration in Group P-5 was significantly greater than those in group P-1 and group C until 48 hours.

* $P < 0.05$ vs. Group C.

# $P < 0.05$ vs. Group P-1.

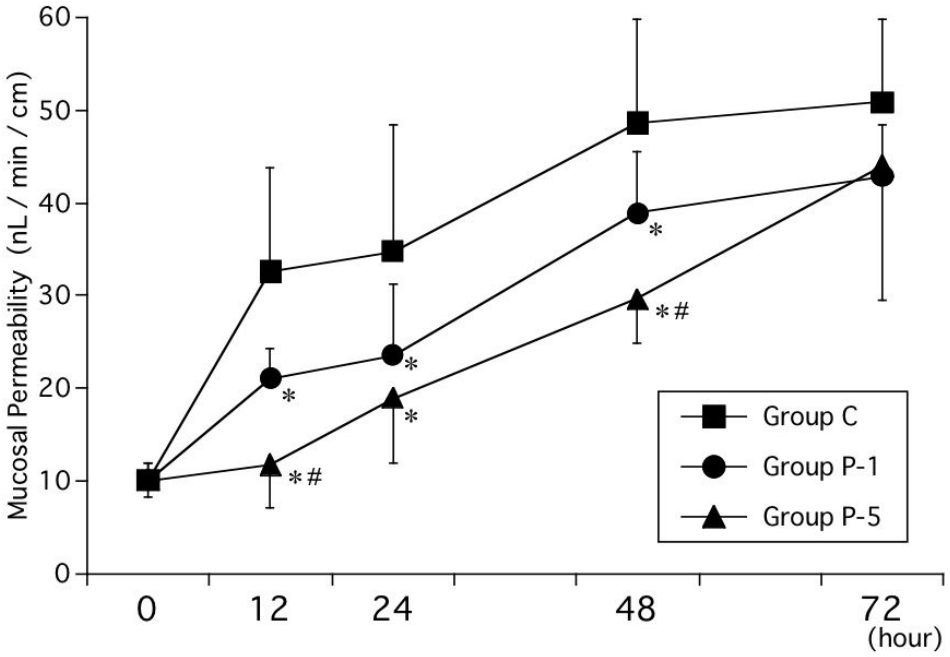


Figure 4.

Changes in mucosal permeability in 5 atm HBO-PFC/UW (Group P-5), 1 atm O2-PFC/UW (Group P-1) and simple cold UW storage (Group C) until 72 hours (n = 10 in each group and time-point). The mucosal permeability in Group P-5 was significantly smaller than those in group P-1 and group C at 12 hours and 48 hours.

* $P < 0.05$ vs. Group C.

# $P < 0.05$ vs. Group P-1.

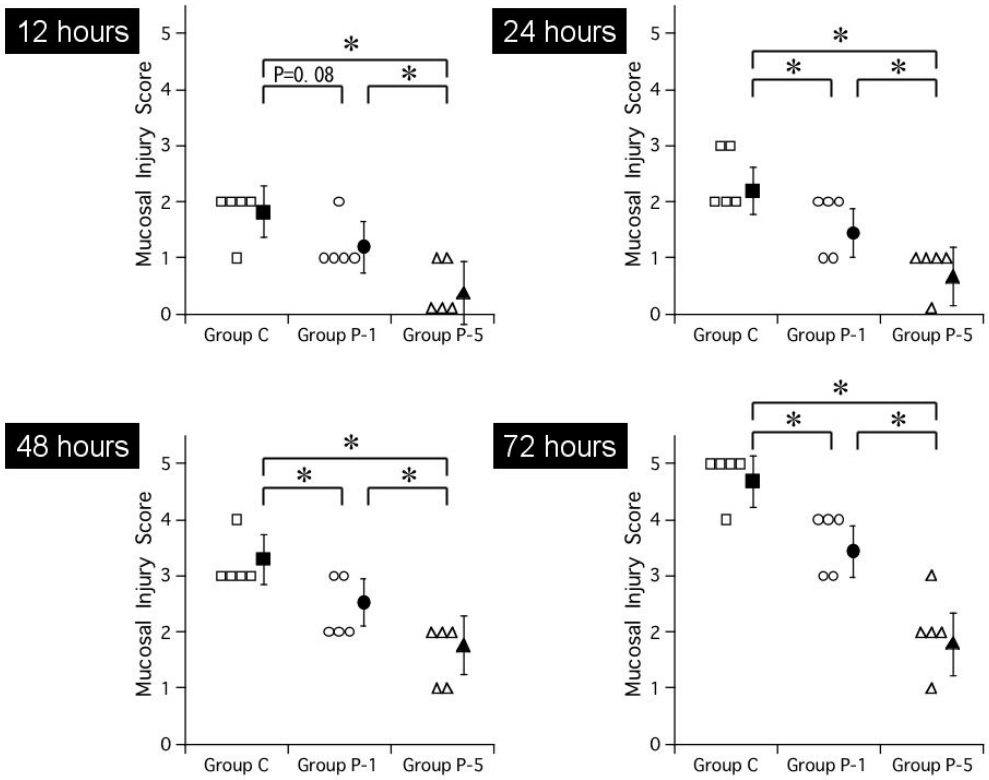


Figure 5.

Histological grading by Chiu's classification (n = 5 in each group and time-point). 5 atm HBO-PFC/UW (Group P-5) showed significant amelioration of histological damage compared with those in 1 atm O2-PFC/UW (Group P-1) and simple cold UW storage (Group C) at each time-point.

* $P < 0.05$ vs. Group C.

# $P < 0.05$ vs. Group P-1.

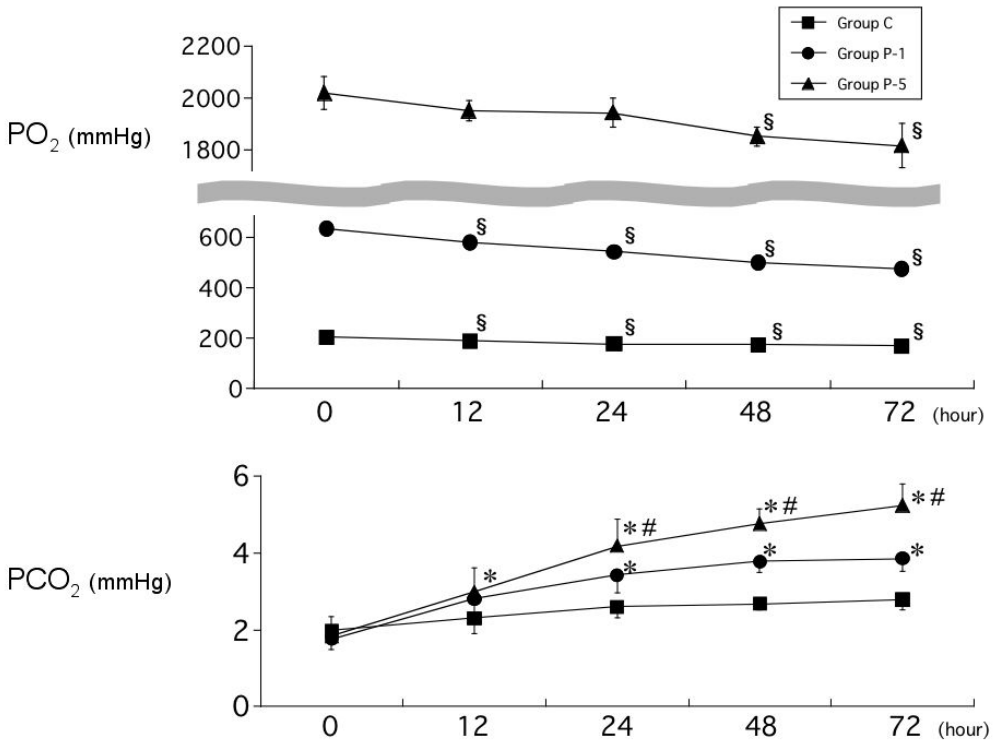


Figure 6.

Changes in PO_2 and PCO_2 in UW solution until 72 hours in Group P-5, Group P-1 and Group C (n = 5 in each group and time-point).

* $P < 0.05$ vs. Group C.

# $P < 0.05$ vs. Group P-1.

§ $P < 0.05$ vs. zero hour.

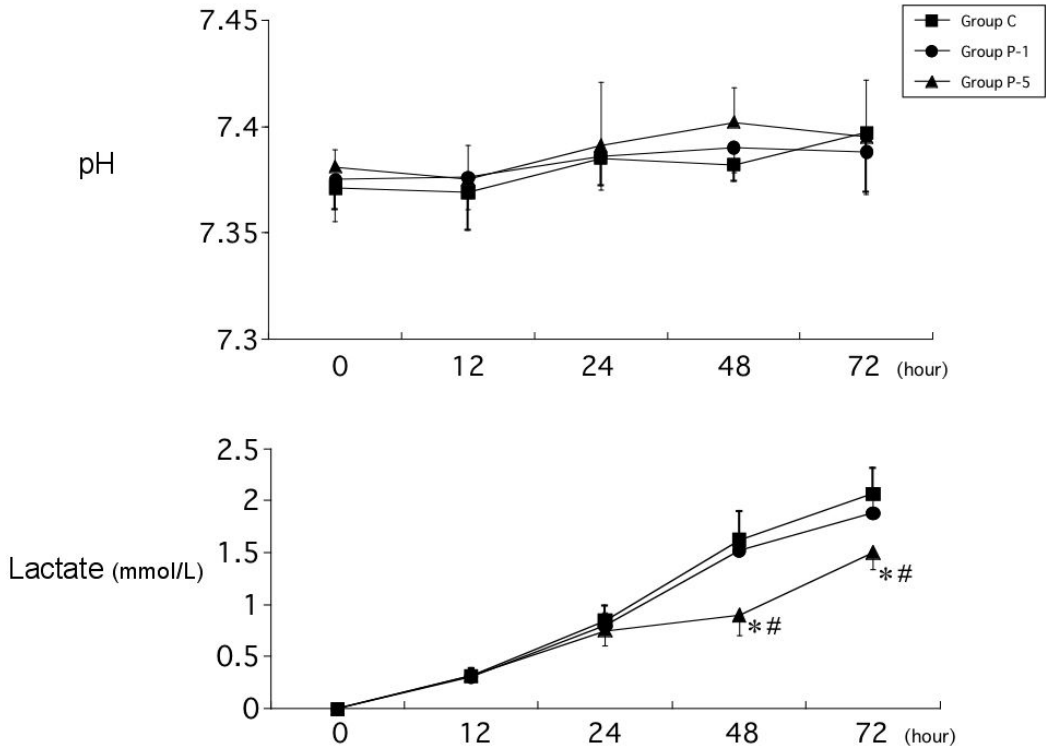


Figure 7.

Changes in pH and Lactate in UW solution until 72 hours in Group P-5, Group P-1 and Group C (n = 5 in each group and time-point).

* $P < 0.05$ vs. Group C.

# $P < 0.05$ vs. Group P-1.